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N1-Src kinase is required for primary neurogenesis in *Xenopus tropicalis*

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Abstract

The presence of the neuronal-specific N1-Src splice variant of the C-Src tyrosine kinase is conserved through vertebrate evolution, suggesting an important role in complex nervous systems. Alternative splicing involving a *N1-Src* specific microexon leads to a five or six amino acid insertion into the SH3 domain of Src. A prevailing model suggests that N1-Src regulates neuronal differentiation via cytoskeletal dynamics in the growth cone. Here we have investigated the role of n1-src in the early development of the amphibian *Xenopus tropicalis*, and find that *n1-src* expression is regulated during embryogenesis, with highest levels detected during the phases of primary and secondary neurogenesis. *In situ* hybridisation analysis, using locked nucleic acid (LNA) oligo probes complementary to the *n1-src* microexon indicate that *n1-src* expression is highly enriched in the open neural plate during neurula stages and in the neural tissue of adult frogs. Given the *n1-src* expression pattern, we investigated a possible role for n1-src in neurogenesis. Using splice site-specific antisense morpholino oligos, we are able to inhibit *n1-src* splicing, whilst preserving *c-src* expression. Differentiation of neurons in the primary nervous system is reduced in *n1-src* knockdown embryos, accompanied by a severely impaired touch response in later development. These data reveal an essential role for n1-src in amphibian neural development and suggest that alternative splicing of C-Src in the developing vertebrate nervous system evolved to regulate neurogenesis.

Significance statement

The Src family of non-receptor tyrosine kinases act in signalling pathways that regulate cell migration, cell adhesion and proliferation. Srcs are also enriched in the brain where they play key roles in neuronal development and neurotransmission.

Vertebrates have evolved a neuron-specific splice variant of C-Src, N1-Src, which differs from C-Src by just five or six amino acids. N1-Src is poorly understood and its high similarity to C-Src has made it difficult to delineate its function. Using antisense knockdown of the *n1-src* microexon, we have studied neuronal development in the *Xenopus* embryo in the absence of *n1-src*, whilst preserving *c-src*. Loss of n1-src causes a striking absence of primary neurogenesis, implicating n1-src in the specification of neurons early in neural development.

Introduction

The Src family of eleven non-receptor tyrosine kinases evolved to regulate key signalling pathways involved in cell adhesion, migration and cell fate in multicellular organisms (Thomas and Brugge, 1997). Several Src family members, including C-Src, Fyn and Yes, are enriched in the vertebrate nervous system with roles in the developing and mature brain and have been implicated in the pathology of neurological disorders (Grant et al., 1992; Maness, 1992; Zhao et al., 2000; Ohnishi et al., 2001; Kalia et al., 2004; Nygaard et al., 2014). Further complexity and specificity of C-Src signalling in the brain, is conferred by neuronal-specific splicing to yield N1- or N2-Src (Brugge et al., 1985; Pyper and Bolen, 1990). The N-Src splice variants contain an additional six or seventeen amino acids respectively in the SH3 domain, and are encoded by microexons situated between exons three and four of C-Src (Martinez et al., 1987). We and others have shown that N-Srcs have a higher constitutive kinase activity and an altered SH3 domain substrate specificity compared to C-Src (Dergai et al., 2010; Keenan et al., 2015), however, their *in vivo* substrates are unknown.

C-Src expression has been identified in a wide range of animal groups, including basal metazoans, such as sea sponges (Ottilie et al., 1992), but its neuronal splicing to yield N1-Src only appears in the vertebrate lineage (Fig. 1A; Levy et al., 1987; Martinez et al., 1987; Raulf et al., 1989) and N2-Src is restricted to mammals (Pyper and Bolen, 1990). Within the N1-Src microexon, there are minor differences in the length and sequence between vertebrate species. For example, a six amino acid N1-Src insert has been detected in brain tissue from the teleost fish *Xiphophorus* (Raulf et al., 1989), whereas the *c-src* locus of the diploid amphibian *Xenopus tropicalis* and the two pseudoallelic loci of allotetraploid *Xenopus laevis* contain five amino acid inserts (Collett and Steele, 1992). Identical six amino acid neuronal Src inserts are observed in N1-Src of chicks, rodents and humans (Levy et al., 1987; Martinez et al., 1987). The appearance and conservation of a neural-restricted src isoform in the vertebrate lineage raises the intriguing possibility that n1-src function is related to the evolution and development of the complex vertebrate nervous system.

Previous studies in which N1-Src was overexpressed suggest N1-Src regulates neuronal morphology through cytoskeletal modifications affecting neurite outgrowth and axonogenesis (Worley et al., 1997; Kotani et al., 2007). However, no studies have thus far observed the development of the nervous system in the absence of N1-Src splicing. Here, we investigated n1-src function in the amphibian *Xenopus tropicalis*. We found that *n1-src* expression is localised to the dorsal ectoderm of the neural plate, which gives rise to the central nervous system during development. Using antisense morpholino oligos, we have for the first time achieved specific inhibition of *n1-src* splicing in a vertebrate nervous system, without affecting *c-src* expression. The knockdown of *n1-src* caused abnormal touch responses in

larval stage embryos, with a concomitant reduction in neuronal-specific tubulin (*tubb2a*) positive neurons during primary neurogenesis. We propose that neuronal splicing of C-Src has evolved to be essential for vertebrate neurogenesis.

Materials and Methods

Sub-cloning of Xenopus n1-src

A plasmid encoding C-terminal FLAG-tagged *Xenopus* n1-src (pFLAG-Xn1-Src) was generated by amplifying the *Xenopus laevis n1-src b* variant open reading frame from IMAGE clone: 5572523 with the following PCR primers incorporating 5' BglII and 3' KpnI restriction sites. This codes for an n1 insertion identical to that of *Xenopus tropicalis* n1-src, as determined by examination of the *Xenopus tropicalis* genome and sequencing of relevant rt-PCR products.

forward 5'-AGATCTCTCTAGAACCATGGGTGCCACTAAAAGCAAGCCA-3'

reverse 5'-GGTACCGTAGATCCAAGGTGTTCCCCAGGCTGGTACTG-3'.

Digested product was ligated into pEGFP-N1 (Clontech, Mountain View, CA) in which the GFP tag was replaced with a FLAG tag (pFLAG). The pCS2+-Xn1-src-FLAG plasmid was generated by excising FLAG-tagged Xn1-src from pFLAG-Xn1-src with XbaI and ligating into XbaI digested pCS2+. The preparation of pFLAG-C-Src and -N1-Src was previously described (Keenan et al., 2015).

Fibroblast cell morphology assay

Ten thousand COS7 fibroblast cells were plated onto 13 mm coverslips. Twenty four hours after plating, cells were transfected with 1 µg plasmid DNA using Ecotransfect (Oz Biosciences) according to the manufacturer's instructions. Cells were fixed 48 h after transfection in 4 % paraformaldehyde, 4 % sucrose for 20 min and then

permeabilised in 0.1 % Triton, 1 % BSA and stained with primary antibodies (mouse anti-FLAG (M2), 1:1000; rabbit anti-GFP, 1:500) in 1 % BSA in PBS for 2 h at room temperature. After 3 washes in PBS, secondary antibodies (anti-mouse Alexa Fluor-564 and anti-rabbit Alexa Fluor-488; Invitrogen, Paisley, UK) were applied at 1:500 in 1 % BSA in PBS for 1 h in the dark. Coverslips were mounted on slides using Mowial mountant (10 % Mowial, 25 % glycerol in 0.1 M Tris pH 8.5) containing 1 µg/ml DAPI. Images were acquired using a 40x objective on a Nikon TE200 epifluorescence inverted microscope using a RoleraXR CCD (QImaging) camera controlled by SimplePCI Software (Hamamatsu). The percentage of COS7 cells bearing neurite-like processes, defined as being longer than the cell soma diameter and having a width of less than 2 µm, was calculated and statistical analysis of the data was performed with SigmaPlot software using a Kruskal-Wallis two tailed analysis of variance. The experimenter was blind to the plasmid transfected in each condition.

Embryological methods

Xenopus tropicalis embryos were produced as previously described (Khokha et al., 2005; Winterbottom et al., 2010). Embryos were microinjected at the 2- or 4-cell stage and cultured at 22ºC in MRS/9+3% Ficoll, before transferring to MRS/20 for long term culture. The sequences for the splice blocking antisense morpholino oligos (GeneTools, LLC) are shown below.

AMO a (splice acceptor) 5'-GTCAGGTCTCCTATGGCACAGCATG-3'

AMO d (splice donor) 5'-GCCGCCGGATGGTCACATACCTCAT-3'

Videos of the locomotive phenotypes of Stage 28 or 41 embryos in response to touch stimuli were acquired using a JVC TK-C1381 camera and processed with ArcSoft ShowBiz software.

RNA extraction and semi-quantitative rt-PCR

Demembranated embryos or tissues dissected from male adult *Xenopus tropicalis* were flash frozen on dry ice. Total RNA was extracted from tissues using Tri-Reagent and precipitated with 7.5 M LiCl/50 mM EDTA (Warrander et al., 2016). First strand cDNA was synthesised from 1-3 µg total RNA using random hexamer primers and Invitrogen SuperScript II Reverse Transcriptase according to the manufacturer's instructions (Warrander et al., 2016). Promega PCR Master Mix was used to amplify cDNA from the embryos at the different developmental stages, with *rpl8* used as a loading control. Primers used to detect gene expression were as follows.

***rpl8* forward**	5'-GGGCTGTCGACTTCGCTGAA-3'
***rpl8* reverse**	5'-ATACGACCACCACCAGCAAC-3'
***c-src* forward**	5'-ATCTCGCACCGAGACAGACT-3
***c-src* reverse**	5'-CAGTCGCCTTCCGTGTTATT-3'
***n1-src* forward**	5'-ACTGTGACCTGACGCCTTTT-3'
***n1-src* reverse**	5'-CCTCATGTCAGGTCTCGTGT-3'.

In situ hybridisation and β-galactosidase staining

Tubb2a (*n-tubulin*) probe synthesis and *in situ* hybridisation were carried as previously described (Winterbottom et al., 2010). A 19-mer digoxigenin end-labelled locked nucleic acid (LNA) probe was designed against the 15 base *n1-sr*c microexon sequence, with the addition of two bases from the flanking *c-src* exons. The probe with the following sequence was synthesised by Exiqon (Vedbaek, Denmark; (Darnell et al., 2010). Locked nucleotides are indicated in bold.

***n1-src* microexon probe** 5'-**T**CC**C**TC**A**TG**T**CA**G**GT**C**TC**G-**3'

It was confirmed that no off-target sequence identities of more than 12 nucleotides were present in the *Xenopus tropicalis* genome.The LNA *in situ* hybridisation was undertaken as previously described (Sweetman, 2011; Warrander et al., 2016). Briefly, de-membraned embryos were fixed in 0.1 M MOPS, 2 mM EDTA, 1 mM MgSO4, 3.7 % formaldehyde. The hybridisation and washes were carried out at 57°C. Embryos were hybridised for 36 h with 20 nM LNA probe preabsorbed again tailbud stage embryos. Colour was developed with BM Purple (Roche) substrate until diffuse purple staining was visible, at which point embryos were washed for 12 h. The staining and washing cycle was then repeated until strong specific staining was present. For lineage tracing, *β-galactosidase* mRNA synthesis, embryo injection and enzyme staining was undertaken as previously described (Pownall et al., 1996). Both *in situs* and fixed phenotypes were imaged using a Leica MZ FLIII microscope (Leica), a SPOT 14.2 Colour Mosaic camera and SPOT Advanced software (Diagnostic Instruments Inc.).

Results

The Xenopus n1-src *splice variant promotes neurite outgrowth*

We first investigated whether the activity of N1-src isoforms has been conserved during vertebrate evolution. There are minor differences in the length and sequence of the *n1-src* microexon between mammals, amphibians and fish, however, the distribution of charged and hydrophobic residues is conserved (Fig 1A). Overexpression of mammalian N1-Src (but not C-Src) was previously shown to elicit morphological changes in *Xenopus* kidney epithelial cells (Worley et al., 1997) and we therefore performed a similar assay to compare the biological activity of *Xenopus* n1-src and mammalian N1-Src (Fig. 1*B,C*). COS7 fibroblasts were co-transfected

with soluble CFP (to aid the visualisation of cell morphology) and a C-terminal FLAG-tagged Src construct (C-, N1- or n1-src) or a vector control. We and others have previously shown that C-terminal fusion tags do not affect Src activity in cells (Sandilands et al., 2004; Keenan et al., 2015). We assayed cell morphology by quantifying the percentage of cells bearing neurite-like processes. In agreement with previous findings, C-Src did not elicit process outgrowth compared to the vector control, while approximately one third of N1-Src and n1-src transfected cells bore processes (Fig. 1*B,C*), suggesting that, despite sequence differences their N1-Src insertions, activities of the amphibian and mammalian N1-Src enzymes have been highly conserved during evolution.

N1-src *is expressed during neurogenesis*

We next examined the temporal expression of *Xenopus n1-src* during development. Using splice variant specific PCR primer sets, we undertook RT-PCR analysis of *c-src* and *n1-src* expression from cleavage to early larval stages (Fig. 2A). Expression of *c-src* is relatively constant throughout early development. In contrast, *n1-src* expression is highly regulated over the same period. Prior to the onset of transcription from the zygotic genome at blastula stage 8, only very low levels of maternally deposited *n1-src* mRNA are detected. Zygotic *n1-src* expression begins to rise at gastrula stage 11, reaching its highest level at neurula stage 18, and this is maintained through early tailbud stage 25. However, by early larval stage 35 expression has fallen dramatically. Fig 2B indicates that *n1-src* expression increases again at stage 46, correlating with secondary neurogenesis of motor, inter- and sensory neurons in the closed neural tube (Schlosser et al., 2002). Therefore, *n1-src* expression is maximal during phases of neurogenesis in the primary nervous system.

We also examined the expression of *c-src* and *n1-src* in adult tissues and found that the highest level of *n1-src* expression is within the adult brain, with heart muscle the only other tissue, where we were able to detect low levels of *n1-src* expression (Fig. 2C).

N1-src *expression is enriched in the neural plate*

To visualise the spatial expression pattern of *n1-src* in the developing embryo we used a 19-mer locked nucleic acid (LNA) probe specific for the *n1-src* microexon sequence. Traditional antisense mRNA *in situ* probes are typically greater than ~150 bases in length and are unable to distinguish between the small sequence differences exhibited by the *c-src* and *n1-src* splice variants. Early and late stage neurula embryos were probed with a digoxigenin labelled *n1-src* specific LNA probe. In keeping with our RT-analysis we find that *n1-src* expression is highly enriched in the neural plate of neurula stage embryos (Fig. 3). Our analysis indicates general expression of *n1-src* in cells of the neural plate at stage 14 (Fig 3A and B). Expression is fairly constant along the anteroposterior axis of the neural plate, with expression being detected in cells of the presumptive fore, mid and hindbrain regions, as well as the spinal cord (Fig 3A, C and D). n1-src expression continues to be enriched in the neural plate as it narrows and rolls up to from the neural tube in late stage 19 neurula embryos (Fig. 3E and F).

Morpholino mediated knockdown of* Xenopus *n1-src disrupts the touch response of embryos

Morpholino oligos (MOs) are nucleic acid analogs with a modified backbone chemistry which are able to hybridize to target RNA in a highly specific, sequence

dependent fashion. Antisense MOs (AMOs) are able to knockdown gene function in a number of systems (Eisen and Smith, 2008). Typical knockdown strategies use AMOs to block translation or nuclear pre-mRNA processing. AMOs targeted to splice acceptor and donor sites in pre-mRNAs have been used to block normal splicing events leading to the formation of aberrant mRNAs containing intron sequences, thus disrupting the protein coding information normally found in the mature mRNA. AMOs have also been successfully used to induce exon skipping (Goyenvalle et al., 2010; Kang et al., 2011). Here we use this approach to block splicing involving the *n1-src* specific microexon. Non-overlapping AMOs targeted to the splice acceptor (AMO a) and donor (AMO d) sites of the *n1-src* microexon were designed (Fig.4A). *n1-src* AMOs were delivered to the cells of the embryo by microinjection. In contrast to uninjected control embryos, we are unable to detect *n1-src* expression in embryos injected with a combination of AMO a+d. Furthermore, injection of AMO a or d alone also effectively blocked *n1-src* expression (Fig. 4B). Consistent with an effect on exon skipping, the expression of *c-src* was unaffected by the AMOs. We conclude that AMOs represent highly specific tools for investigating the function of the *Xenopus* n1-src isoform in early development.

Injection of a standard control MO has little effect on the phenotype of larval stage 41 embryos, whereas injection of the AMO a+d mixture leads to a mild, but highly penetrant phenotype, which is characterised by shortening and/ or kinking of the tail, and variable disruption to the pigmented retina of the eye (Fig. 4C). To assess the function of the primary nervous system in *n1-src* ablated embryos, we applied a touch stimulus to the side of larval stage embryos, which elicits a dart response. The neuronal circuitry for the touch reflex (Fig. 4D; Movie 1) is well characterised and comprises Rohon-Beard sensory neurons, which activate

commissural interneurons that in turn synapse onto contralateral motor neurons to stimulate muscle contraction, propelling the embryo away from the stimulus (Boothby and Roberts, 1995; Li et al., 2003; Roberts et al., 2010). (Fig 4D; Movie 1, 3). We tested the same embryos at developmental stages 28 and 41 (prior to and during the onset of myelination) and the dart response was commonly abnormal or absent in *n1-src* AMO a+d injected embryos (Fig. 4E). These embryos instead frequently displayed an uncoordinated twitch or spasm response, indicating abnormal development of the neural circuitry necessary for the dart response (Fig 4D; Movie 2, 4).

N1-src knockdown disrupts primary neurogenesis

To ascertain which neurons in the touch reflex are affected by *n1-src* knockdown, we next investigated the early development of the primary nervous system. During primary neurogenesis, the motor, inter and Rohon-Beard sensory neurons differentiate to form medial, intermediate and lateral columns respectively, either side of the neural plate midline. These columns of differentiating neurons are separated by non-differentiating, proliferative progenitors and can be identified by expression of the neuronal specific *tubb2b* gene (Chitnis et al., 1995). Fig. 4F shows differentiating primary neurons visualised by *in situ* hybridization for *tubb2b* mRNA. Unilateral injection of AMO a+d resulted in penetrant reduction of *tubb2b* expression in all three columns relative to the uninjected contralateral side. (90%, n=55 from four independent fertilisations) or embryos unilaterally injected with a control MO (24%, n=39 from four independent fertilisations). Thus we concluded that rather than regulating the development of specific subsets of neurons, *n1-src* is required for

neurogenesis of all *tubb2b*-positive neurons in *Xenopus* primary nervous system development.

Discussion

The activity of amphibian and mammalian n1-src is conserved

The alternative splicing of neuronal src isoforms alters the ligand binding specificity of the C-Src SH3 domain and the catalytic activity of its kinase domain (Brugge et al., 1985; Keenan et al., 2015). These differences are believed to underpin the reported differential activity of neuronal Srcs. The position of the N1-specific insertion into the SH3 domain of C-Src is conserved between amphibians and amniotes, and we investigated whether the differential biological activity of N1-Src isoforms has been conserved in amphibians. We find that, unlike C-Src, both *Xenopus* n1-src, and mammalian N1-Src, despite different SH3 inserts (5 versus 6 amino acid in amphibians and mammals respectively), are able to induce neurite-like processes when transfected into COS-7 cells. In keeping with this, it has been shown previously that N1-Src overexpression in *Xenopus A6 e*pithelial cells, induced neurite-like processes, in contrast to the rounded phenotype of C-Src transfected cells (Worley et al., 1997).

n1-src expression correlates with primary neurogenesis

A previous study indicated that the expression of the *n1-src* isoforms of the tetraploid amphibian *Xenopus laevis*, are initiated by mid-neurula stage 15 (Collett and Steele, 1992). We find that in the diploid amphibian *Xenopus tropicalis*, there is low level maternal *n1-src* expression from the start of development, and, in contrast to the previous study, activation of zygotic *n1-src* expression is initiated as early as mid to

late gastrula stages, and by early neurula stages expression is restricted to the open neural plate.

The period from late gastrula to early neurula is a key phase in the development of the primary nervous system; a simple functional nervous system, characteristic of anamniotic aquatic vertebrates, including fish and amphibians (Hartenstein, 1989). Differentiation of primary neurons enables the early development of motility, and helps embryos avoid predation in an aquatic environment. Primary neurons begin to differentiate at open neural plate stages in *Xenopus* embryos and primary neurogenesis continues through neurula and early tailbud stages (Schlosser et al., 2002). In keeping with our findings that n1-src expression is initiated during the gastrula stage, a subsequent study by Collet and Steele (1993) showed that *n1-src* expression is rapidly activated in competent gastrula dorsal ectoderm by endogenous neural inducing signals and the neural inducing activity of the phorbol ester TPA in the absence of protein synthesis.

Abnormal neural development in n1-src knockdown embryos

We present the first analysis of the consequences of blocking the splicing events required for the expression of *n1-src* during vertebrate development. An advantage of our approach is that the morpholinos ablated n1-src expression whilst c-src expression was unaffected. N1-src knockdown caused striking behavioural, morphological and neuronal phenotypes in the *Xenopus* embryo. At the larval stage we found that AMO injected embryos exhibited a severely abnormal locomotor response to touch stimuli, with many observed to twitch or spasm following the touch. The *Xenopus* touch reflex and subsequent swimming circuits involve the co-ordination of sensory, inter and motor neurons (Roberts et al., 2010). In stage 14

323 embryos, and in keeping with the widespread expression of *n1-src* in the neural plate,
324 we found the columns of differentiating *tubb2a*-positive neurons that subsequently
325 form the touch and swimming circuits are reduced or absent. Due to the labile nature
326 of the morpholinos, we predict that n1-src expression will slowly return, leading to a
327 delay and perturbation in primary neurogenesis that generates the aberrant circuits
328 observed in the larval embryo. The reduced touch response could also result from a
329 defect in myelination, a process that begins at approximately stage 42 (Yoshida,
330 1997). Furthermore, oligodendrocytes arise from the same precursors as motor
331 neurons (Park et al., 2002). However, our observation that both stage 28 and stage
332 41 embryos exhibit the same defects rules out myelination as the sole cause of the
333 phenotype.

334 Morphologically, n1-src knockdown embryos exhibited a loss of retinal
335 pigmentation and a kinked tail. The optic stalk, retina and retinal pigmented
336 epithelium develop from an outpocketing of the diencephalon (Fuhrmann et al.,
337 2014). Therefore, the loss of eye pigmentation in n1-src knockdown embryos may
338 indicate a common role for n1-src in regulating the differentiation of cells derived from
339 the neuroepithelium. The morphogenesis of the vertebrate main body axis involves
340 coordinated cell movements in the axial mesoderm and the neuroepithelium
341 (Nikolopoulou et al., 2017). Posterior axial defects have been observed in embryos in
342 which normal convergent extension within the neuroepithelium has been inhibited
343 through interference with components of the planar cell polarity signalling pathway
344 (Goto and Keller, 2002). We speculate that the kinking of the posterior axis observed
345 in n1-src knockdown embryos arises through deregulation of the process of
346 convergent extension, which drives elongation and narrowing of the neuroepithelium

and reflects abnormal signalling within the neuroepithelium in the absence of n1-src activity.

At present we are unable to say with any confidence where n1-src functions in the pathway leading to neuronal differentiation. However, n1-src's general neural expression is similar to a group of neural stabilization genes, including members of the Sox, Zic and Iroquois families (reviewed by Moody and Je, 2002). These code for transcription factors and, as is the case with n1-src, several of these genes are expressed in response to neural induction. Neural stabilization genes have multiple overlapping functions, providing a link between the signals that induce the neural plate and the hierarchy of proneural and neurogenic genes that are required for neuronal specification and differentiation. Thus, early expressed neural stabilization genes have roles in regulating the competence of ectodermal cells to respond to neural inducing signals, and later expressed ones regulate the progression from neuronal progenitor to differentiated neuron (Moody and Je, 2002). Current evidence is suggestive of a role for n1-src in the process of neural stabilization. However, future studies will be required to investigate the regulatory interactions between the n1-src kinase and known components of the vertebrate neurogenic pathway.

N1-Src function and neurogenesis in higher vertebrates

Our data show that following primary neurogenesis *n1-src* expression falls, but is again elevated during a second phase of neurogenesis in late larval stages. A primary nervous system is absent in amniotes, and it is the later phase of secondary neurogenesis in the closed neural tube that is more akin to the neurogenesis of amniotes, including mice and humans (Wullimann et al. 2005). A connection between neuronal differentiation and N1-Src function in amniotes is supported by an analysis

of N1-Src (also termed pp60^{+}) activity in the developing mouse brain, which showed a rapid increase in N1-Src activity at E12 and which peaks at E18, when increasing numbers of neuroblasts are exiting the cell cycle and differentiating (Wiestler and Walter, 1988). Furthermore, cultured neurons of the rat striatum contain little detectable N1-Src activity, however, neuronal differentiation induced by serum starvation leads to a seven-fold increase in N1-Src activity relative to C-Src (Cartwright et al., 1987). Similarly, embryonic carcinoma cells treated with retinoic acid to induce neuronal differentiation express increased levels of N1-Src (Lynch et al., 1986), and there is an increase in both N1-Src and N2-Src expression during differentiation of the neuroblastoma cell line LAN-5 (Matsunaga et al., 1993a).

We present evidence for an early role for n1-src in neural development regulating the transition from neural progenitors to differentiated neuron. However, there is also evidence that N1-Src has roles regulating the cellular architecture and morphogenesis of neurons. Transgenic mice expressing N1-Src in Purkinje neurons of the cerebellum display defects in migration and dendrite morphology, which might be linked to defects in microtubule structure (Kotani et al., 2007). Conversely, in *Xenopus laevis*, overexpression of mammalian N1-Src in the optic tectum enhanced axonogenesis of retinal progenitors. Thus n1-src is likely to have multiple roles in neural development regulating neuronal specification and morphogenesis.

References

Boothby KM, Roberts A (1995) Effects of site of tactile stimulation on the escape swimming responses of hatchling Xenopus laevis embryos. J Zool 235:113–125.

Brugge JS, Cotton PC, Queral AE, Barrett JN, Nonner D, Keane RW (1985)

396 Neurones express high levels of a structurally modified, activated form of pp60c-src.
397 Nature 316:554–557.

398 Chitnis A, Henrique D, Lewis J, Ish-Horowicz D, Kintner C (1995) Primary
399 neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila
400 neurogenic gene Delta. Nature 375:761–766.

401 Collett JW, Steele RE (1992) Identification and developmental expression of Src+
402 mRNAs in Xenopus laevis. Dev Biol 152:194–198.

403 Darnell DK, Stanislaw S, Kaur S, Antin PB (2010) Whole mount in situ hybridization
404 detection of mRNAs using short LNA containing DNA oligonucleotide probes. RNA
405 16:632–637.

406 Dergai M, Tsyba L, Dergai O, Zlatskii I, Skrypkina I, Kovalenko V, Rynditch A (2010)
407 Microexon-based regulation of ITSN1 and Src SH3 domains specificity relies on
408 introduction of charged amino acids into the interaction interface. Biochem Biophys
409 Res Commun 399:307–312.

410 Eisen JS, Smith JC (2008) Controlling morpholino experiments: don't stop making
411 antisense. Development 135:1735–1743.

412 Fuhrmann S, Zou C, Levine EM (2014) Retinal pigment epithelium development,
413 plasticity, and tissue homeostasis. Exp Eye Res 123:141–150.

414 Goto T, Keller R (2002) The planar cell polarity gene strabismus regulates
415 convergence and extension and neural fold closure in Xenopus. Dev Biol 247:165–
416 181.

417 Goyenvalle A, Babbs A, Powell D, Kole R, Fletcher S, Wilton SD, Davies KE (2010)

418 Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient
419 mice by morpholino-oligomer-mediated exon-skipping. Mol Ther 18:198–205.

420 Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER (1992) Impaired long-
421 term potentiation, spatial learning, and hippocampal development in fyn mutant mice.
422 Science 258:1903–1910.

423 Hartenstein V (1989) Early neurogenesis in Xenopus: the spatio-temporal pattern of
424 proliferation and cell lineages in the embryonic spinal cord. Neuron 3:399–411.

425 Kalia LV, Gingrich JR, Salter MW (2004) Src in synaptic transmission and plasticity.
426 Oncogene 23:8007–8016.

427 Kang JK, Malerba A, Popplewell L, Foster K, Dickson G (2011) Antisense-induced
428 myostatin exon skipping leads to muscle hypertrophy in mice following octa-
429 guanidine morpholino oligomer treatment. Mol Ther 19:159–164.

430 Keenan S, Lewis PA, Wetherill SJ, Dunning CJR, Evans GJO (2015) The N2-Src
431 neuronal splice variant of C-Src has altered SH3 domain ligand specificity and a
432 higher constitutive activity than N1-Src. FEBS Lett 589:1995–2000.

433 Khokha MK, Yeh J, Grammer TC, Harland RM (2005) Depletion of three BMP
434 antagonists from Spemann's organizer leads to a catastrophic loss of dorsal
435 structures. Dev Cell 8:401–411.

436 Kotani T, Morone N, Yuasa S, Nada S, Okada M (2007) Constitutive activation of
437 neuronal Src causes aberrant dendritic morphogenesis in mouse cerebellar Purkinje
438 cells. Neurosci Res 57:210–219.

439 Levy JB, Dorai T, Wang LH, Brugge JS (1987) The structurally distinct form of pp60c-

440 src detected in neuronal cells is encoded by a unique c-src mRNA. Mol Cell Biol
441 7:4142–4145.

442 Li W-C, Soffe SR, Roberts A (2003) The spinal interneurons and properties of
443 glutamatergic synapses in a primitive vertebrate cutaneous flexion reflex. J Neurosci
444 23:9068–9077.

445 Maness PF (1992) Nonreceptor protein tyrosine kinases associated with neuronal
446 development. Dev Neurosci 14:257–270.

447 Martinez R, Mathey-Prevot B, Bernards A, Baltimore D (1987) Neuronal pp60c-src
448 contains a six-amino acid insertion relative to its non-neuronal counterpart. Science
449 237:411–415.

450 Moody SA, Je H-S (2002) Neural induction, neural fate stabilization, and neural stem
451 cells. ScientificWorldJournal 2:1147–1166.

452 Nikolopoulou E, Galea GL, Rolo A, Greene NDE, Copp AJ (2017) Neural tube
453 closure: cellular, molecular and biomechanical mechanisms. Development 144:552–
454 566.

455 Nygaard HB, van Dyck CH, Strittmatter SM (2014) Fyn kinase inhibition as a novel
456 therapy for Alzheimer's disease. Alzheimers Res Ther 6:8.

457 Ohnishi H, Yamamori S, Ono K, Aoyagi K, Kondo S, Takahashi M (2001) A src family
458 tyrosine kinase inhibits neurotransmitter release from neuronal cells. Proc Natl Acad
459 Sci U S A 98:10930–10935.

460 Ottilie S, Raulf F, Barnekow A, Hannig G, Schartl M (1992) Multiple src-related
461 kinase genes, srk1-4, in the fresh water sponge Spongilla lacustris. Oncogene

462 7:1625–1630.

463 Park H-C, Mehta A, Richardson JS, Appel B (2002) olig2 is required for zebrafish
464 primary motor neuron and oligodendrocyte development. Dev Biol 248:356–368.

465 Pownall ME, Tucker AS, Slack JM, Isaacs HV (1996) eFGF, Xcad3 and Hox genes
466 form a molecular pathway that establishes the anteroposterior axis in Xenopus.
467 Development 122:3881–3892.

468 Pyper JM, Bolen JB (1990) Identification of a novel neuronal C-SRC exon expressed
469 in human brain. Mol Cell Biol 10:2035–2040.

470 Raulf F, Robertson SM, Schartl M (1989) Evolution of the neuron-specific alternative
471 splicing product of the c-src proto-oncogene. J Neurosci Res 24:81–88.

472 Roberts A, Li W-C, Soffe SR (2010) How neurons generate behavior in a hatchling
473 amphibian tadpole: an outline. Front Behav Neurosci 4:16.

474 Sandilands E, Cans C, Fincham VJ, Brunton VG, Mellor H, Prendergast GC, Norman
475 JC, Superti-Furga G, Frame MC (2004) RhoB and actin polymerization coordinate
476 Src activation with endosome-mediated delivery to the membrane. Dev Cell 7:855–
477 869.

478 Schlosser G, Koyano-Nakagawa N, Kintner C (2002) Thyroid hormone promotes
479 neurogenesis in the Xenopus spinal cord. Dev Dyn 225:485–498.

480 Sweetman D (2011) In situ detection of microRNAs in animals. Methods Mol Biol
481 732:1–8.

482 Thomas SM, Brugge JS (1997) Cellular functions regulated by Src family kinases.

483 Annu Rev Cell Dev Biol 13:513–609.

484 Warrander F, Faas L, Kovalevskiy O, Peters D, Coles M, Antson AA, Genever P,
485 Isaacs HV (2016) lin28 proteins promote expression of 17~92 family miRNAs during
486 amphibian development. Dev Dyn 245:34–46.

487 Winterbottom EF, Illes JC, Faas L, Isaacs HV (2010) Conserved and novel roles for
488 the Gsh2 transcription factor in primary neurogenesis. Development 137:2623–2631.

489 Worley TL, Cornel E, Holt CE (1997) Overexpression of c-src and n-src in the
490 developing Xenopus retina differentially impairs axonogenesis. Mol Cell Neurosci
491 9:276–292.

492 Yoshida M (1997) Oligodendrocyte maturation in Xenopus laevis. J Neurosci Res
493 50:169–176.

494 Zhao W, Cavallaro S, Gusev P, Alkon DL (2000) Nonreceptor tyrosine protein kinase
495 pp60c-src in spatial learning: synapse-specific changes in its gene expression,
496 tyrosine phosphorylation, and protein-protein interactions. Proc Natl Acad Sci U S A
497 97:8098–8103.

Figure legends

Figure 1. *Xenopus* n1-src elicits neurite-like processes in fibroblasts.

A, Amino acid alignment of the N1-microexon in mammalian, *Xenopus,* and fish species. +=basic; -=acidic and Φ=hydrophobic amino acid sidechains. **B**, Representative COS7 cells co-transfected for four days with Src-FLAG and CFP constructs. Cells were stained for Src (anti-FLAG) and CFP. **C***,* Quantification of process outgrowth in COS7 cells. Each process was defined as an extension longer than one cell soma diameter and less than 2 µm in diameter. Data are plotted as mean ± SEM, n=3 independent experiments. Kruskal-Wallis two-tailed analysis of variance. ***, P<0.001. Scale bar = 10 µm.

Figure 2. *n1-src* mRNA expression levels during *Xenopus tropicalis* development and in adult tissues.

A, rt-PCR analysis of *c-src* and *n1-src* mRNA expression levels from early cleavage stage 4 to tailbud stage 35. *rpl8* is used as a ubiquitously expressed loading control. -rt= no reverse transcriptase control and water= no template control. **B**, rt-PCR analysis of *c-src* and *n1-src* expression levels during (stage 25) and after (stage 35) primary neurogenesis, and during secondary neurogenesis (stage 46). **C**, rt-PCR analysis of c-src and n1-src expression in a range of adult tissues.

Figure 3. Expression pattern of *n1-src* during *Xenopus tropicalis* primary neurogenesis.

In situ hybridisation analysis of *n1-src* mRNA expression using a 19-mer digoxigenin end-labelled antisense probe directed against *n1-src* specific sequence. **A**, **B**, **C** and **D** are early neurula stage 14 embryos. **E** and **F** are late neurula stage 19 embryos. **A**,

dorsal view, anterior to left. **B**, lateral view anterior to the left. **C**, anterior view, dorsal to the top. **D**, posterior view, dorsal to the top. **E**, dorsal view anterior to the left. **F**, anterior view dorsal to the top. *F+mb* = presumptive forebrain and midbrain, *sc* = presumptive spinal cord, *bp* = blastopore.

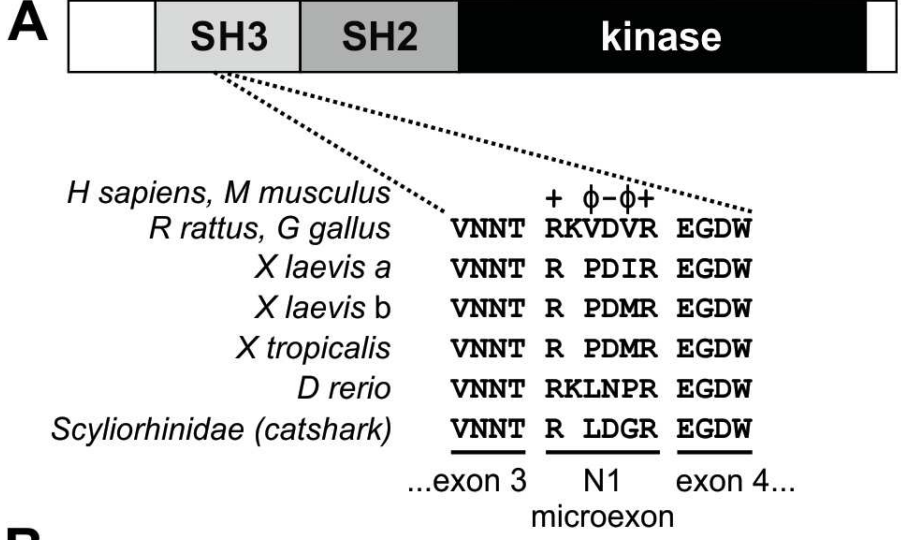
Figure 4. Abnormal touch response and primary neurogenesis in n1-src knockdown embryos

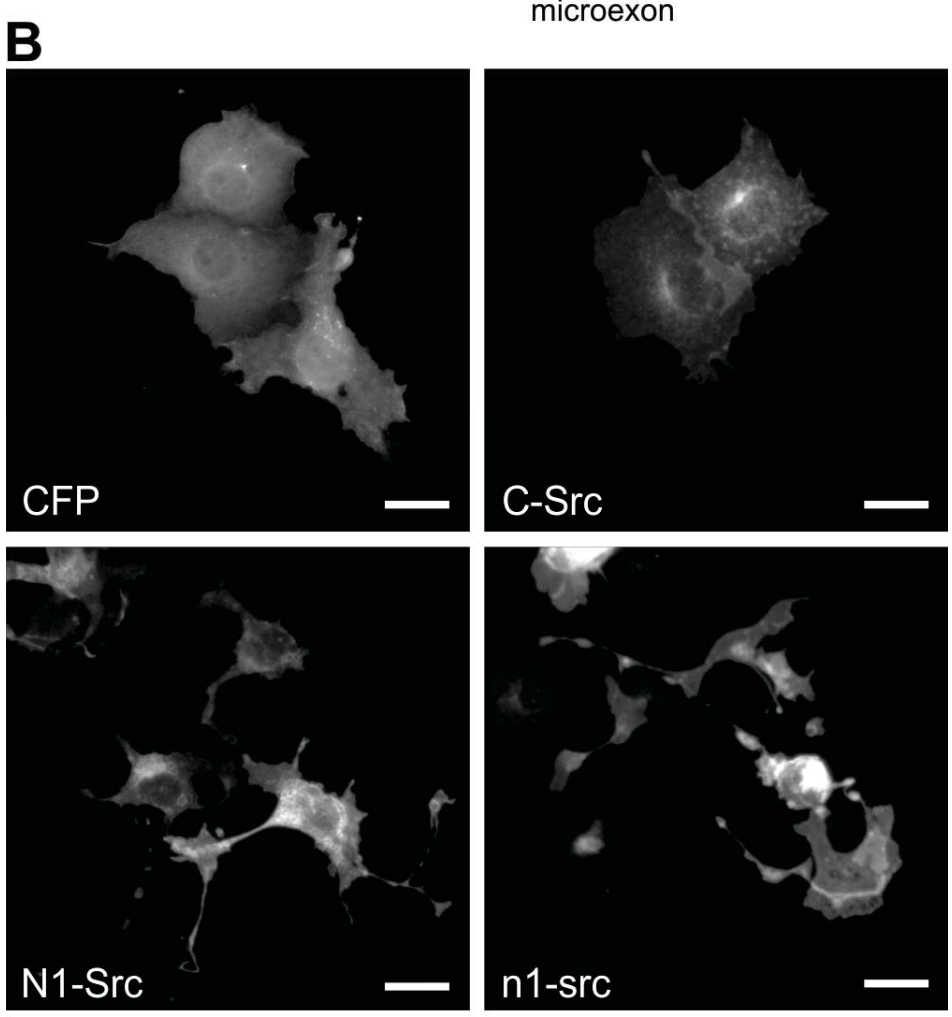
A, diagram showing the sequences and corresponding RNA target sequences of the splice acceptor (AMO a) and donor (AMO d) splice blocking antisense morpholinos. **B**, rt-PCR analysis of *c-src* and *n1-src* mRNA expression at stage 16 in control uninjected and injected with a total of 20 ng of AMO a, AMO d or AMO a+d. *rpl8* is used as a ubiquitously expressed loading control. -rt = no reverse transcriptase control and water = no template control. **C**, representative phenotypes of embryos at larval stage 41 bilaterally injected at the 2- or 4-cell stage with 10 ng total of a standard control MO or the AMO a+d combination. Embryos were co-injected with 100 pg nuclear *β-galactosidase* and subsequently stained with X-gal (light blue colour) to demonstrate successful injection targeting. **D**, cartoon of embryo touch reflex. Touching the skin stimulates Rohon-Beard sensory neurons (s), which synapse onto commissural interneurons (i) that activate contralateral motor neurons (m), leading to muscle contraction. **E,** Quantitation of touch response phenotype of the same embryos at larval stage 28 and 41 bilaterally injected at the 2- or 4-cell stage with 10 ng total of a standard control MO or the AMO a+d combination. Data are plotted as mean ± SEM, n=4 independent experiments. **F**, *in situ* hybridisation analysis of *tubb2b* expression in differentiating primary neurons of open neural plate stage 14 embryos unilaterally injected with 5 ng total of a standard control MO or the

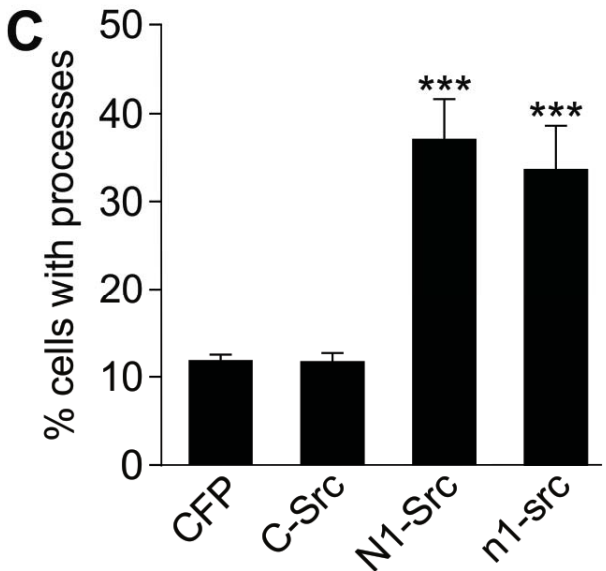
AMO a+d combination. The injected side shows faint blue nuclear staining with the *β-galactosidase* lineage tracer, and is indicated with a black asterisk, anterior is to the left. m=motor neurons; i=interneurons; s=sensory neurons.

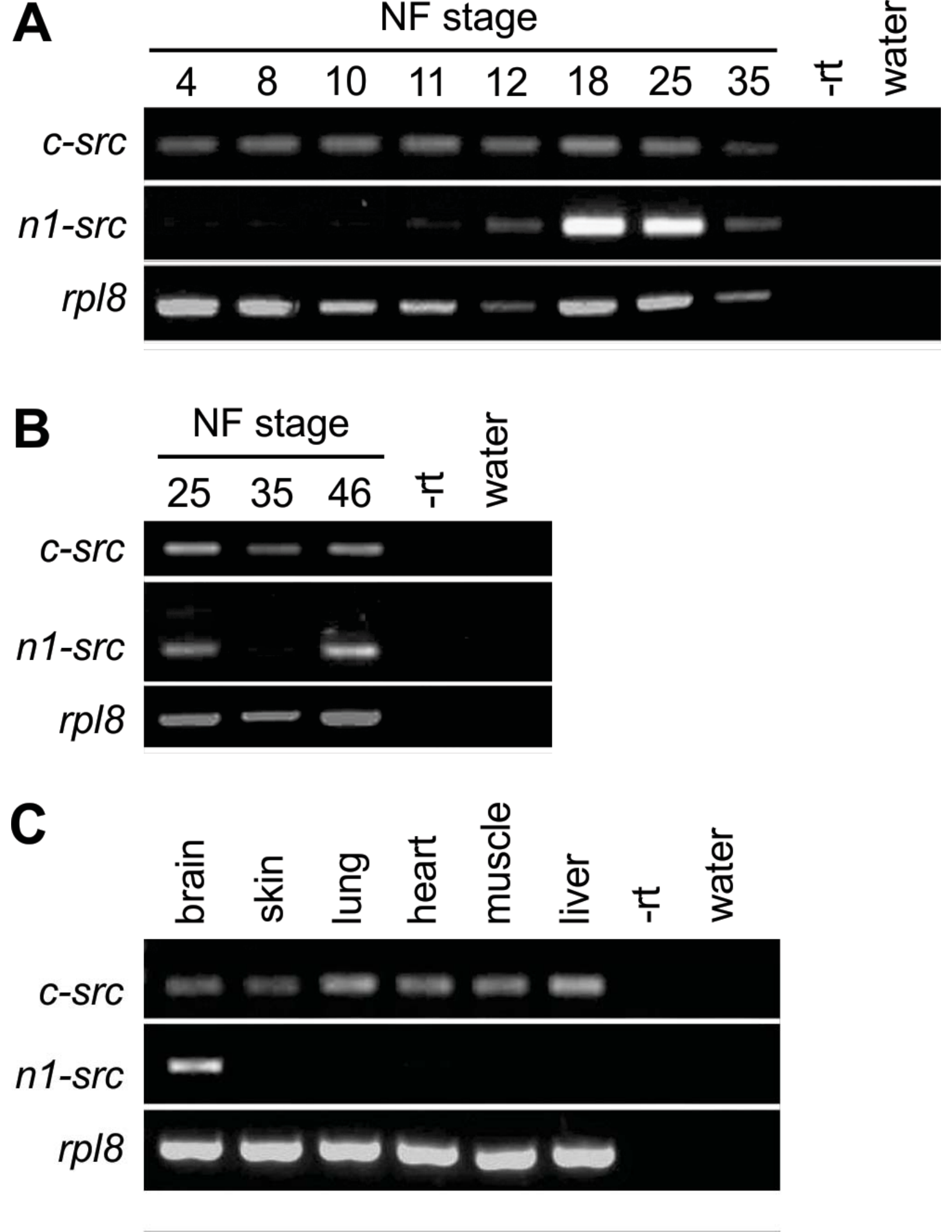
Movies 1-4. Normal and abnormal touch responses in stage 28 and stage 41 *Xenopus tropicalis* embryos.

Movies 1 & 3, real time videos showing the normal touch response of stage 28 (Movie 1) or stage 41 (Movie 3) embryos injected with 10 ng of a standard control MO. Embryos right themselves and swiftly swam a short distance from the point of contact. **Movies 2 & 4**, real time videos of the abnormal touch response in stage 28 (Movie 2) or stage 41 (Movie 4) embryos injected with 10 ng *n1-src* AMO a+d. Non-responding phenotypes remain horizontal and moved slowly from the point of contact with uncoordinated twitching movements.

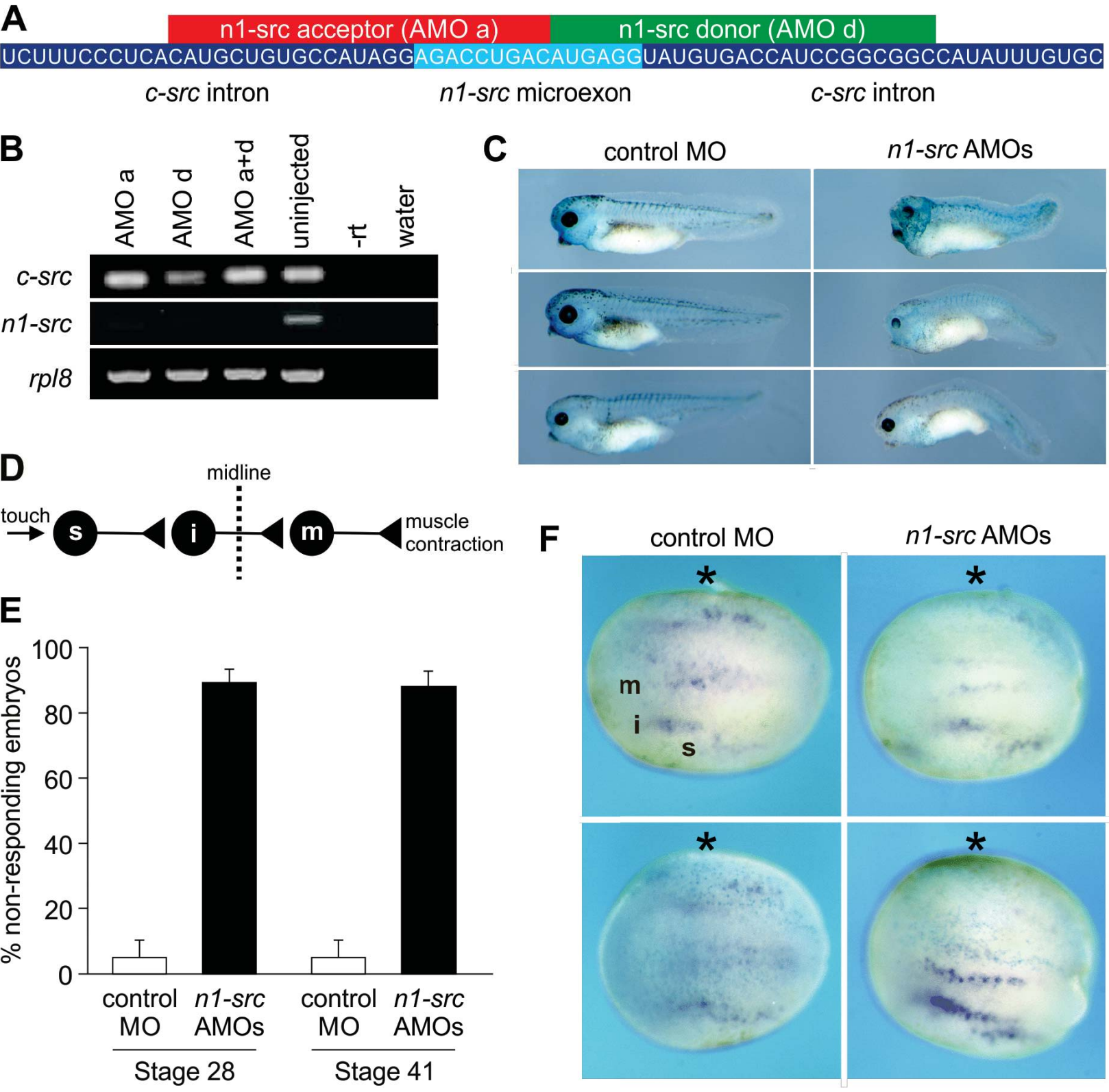

A
SH3
SH2
kinase
H sapiens, M musculus
R rattus, G gallus
X laevis a
X laevis b
X tropicalis
D rerio
Scyliorhinidae (catshark)
+ ϕ−ϕ+
VNNT RKVDVR EGDW
VNNT R PDIR EGDW
VNNT R PDMR EGDW
VNNT R PDMR EGDW
VNNT RKLNPR EGDW
VNNT R LDGR EGDW
...exon 3
N1
microexon
exon 4...


B
CFP
C-Src
N1-Src
n1-src


C
% cells with processes
50
40
30
20
10
0
***
***
CFP
C-Src
N1-Src
n1-src


A
NF stage
4
8
10
11
12
18
25
35
-rt
water
c-src
n1-src
rpl8
B
NF stage
25
35
46
-rt
water
c-src
n1-src
rpl8
C
brain
skin
lung
heart
muscle
liver
-rt
water
c-src
n1-src
rpl8

A
f+mb
hb
sc
dorsal
B
lateral
C
anterior
D
bp
posterior
E
dorsal
F
anterior
early neurula
(stage 14)
late neurula
(stage 19)


A
n1-src acceptor (AMO a)
n1-src donor (AMO d)
UCUUUCCCUCACAUGCUGUGCCAUAGGAGACCUGACAUGAGGUAUGUGACCAUCCGGCGGCCAUAUUUGUGC
c-src intron
n1-src microexon
c-src intron
B
AMO a
AMO d
AMO a+d
uninjected
-rt
water
c-src
n1-src
rpl8
C
control MO
n1-src AMOs
D
midline
touch
s
i
m
muscle contraction
E
% non-responding embryos
100
80
60
40
20
0
control MO
n1-src AMOs
control MO
n1-src AMOs
Stage 28
Stage 41
F
control MO
n1-src AMOs
m
i
s